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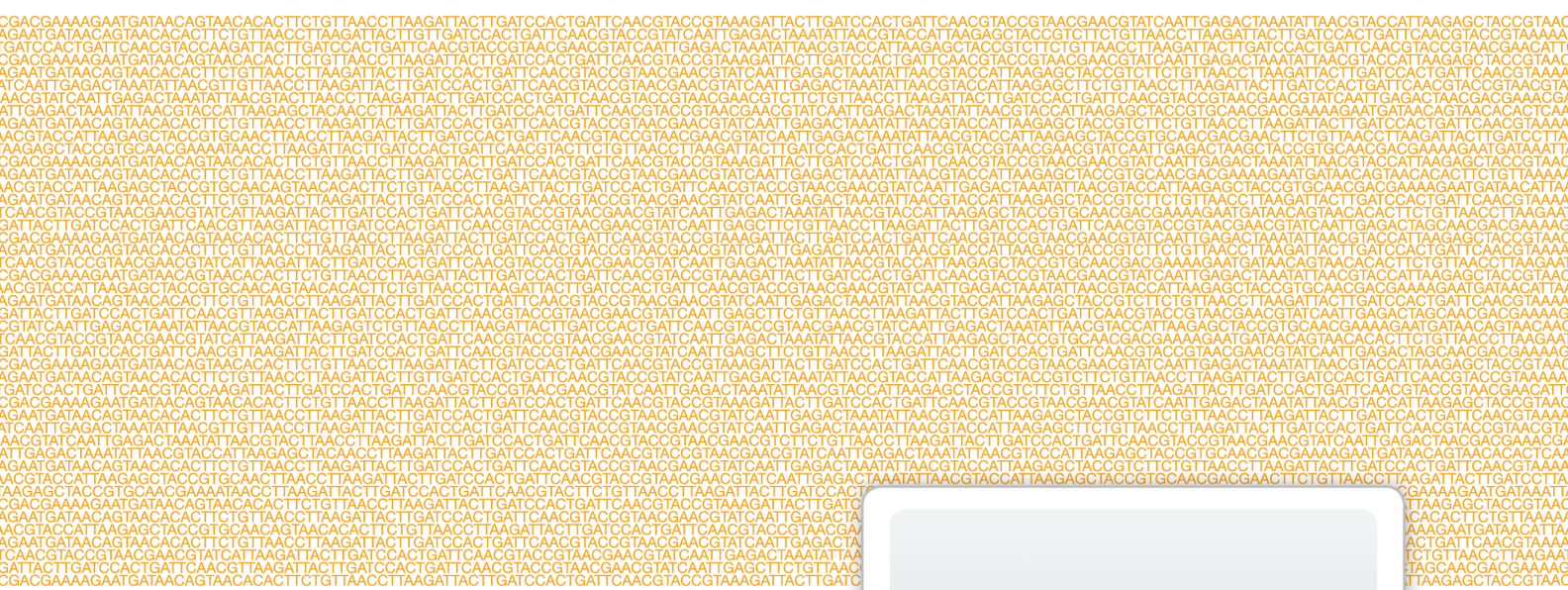
TruSeq® Custom Amplicon Library Preparation Guide



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Revision History

Part #	Revision	Date	Description of Change
15027983	C	August 2013	The following changes were made to the user guide: • Reorganized Getting Started content and moved some topics to the *Supporting Information* Appendix. • Introduction of reagents OHS2, ELM4, and LNS2. • Revised DNA input and volume recommendations for high-quality genomic DNA. See *DNA Input Recommendations* on page 3. • Revised instructions for hybridization using OHS2. • Revised PCR cycling conditions for DNA input <100 ng. See *PCR Amplification* on page 17. • Revised instructions for preparation of DAL and sample pooling recommendations in *Library Pooling and MiSeq Sample Loading* on page 34 to support MiSeq Reagent Kit v3.

Part #	Revision	Date	Description of Change
15027983	B	September 2012	The following changes were made to the user guide: • Revised DNA input recommendations for high-quality genomic DNA and recommendations for the use of FFPE DNA. See *DNA Input Recommendations* on page 3. • Updated filter plate pre-wash and wash recommendations for removal of unbound oligos. See *Removal of Unbound Oligos* on page 12. • Revised instructions for removal of LNW1 supernatant after library normalization. See *Library Normalization* on page 29. • Revised instructions to specify required workflows when using the Illumina Experiment Manager. See *MiSeq Sample Sheet Preparation* on page 52. • Overview of the TSCA process, from design to data analysis, including a new section for the *Illumina Amplicon Viewer* on page 54. See *TruSeq Custom Amplicon Library Preparation Process Overview* on page 42. • Expanded assay conditions to support new amplicon sizes, FFPE DNA, and various target multiplexing levels. See *PCR Amplification* on page 17 and *PCR Clean-Up* on page 23.
15027983	A	October 2011	Initial release

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Overview

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Introduction

TruSeq Custom Amplicon Library Preparation (TSCA) allows researchers to sequence targeted regions of the genome spanning upwards of 600 kb with up to 1,536 amplicons in a single multiplex reaction. This highly targeted approach enables a wide range of applications for discovering, validating, and screening genetic variants in a rapid and efficient manner. TSCA enables a high level of multiplexing by generating up to 1,536 amplicons within a single reaction and integrated indices support sequencing up to 96 samples per MiSeq run. TSCA leverages the long paired-end read capability, speed, and high data quality of the MiSeq System.

Excellent Multiplexing Capability

- Amplify up to 1,536 amplicons in a single reaction and sequence up to 96 samples in a single MiSeq run.

Revolutionary Assay with Fast and Simple Workflow

- Generate up to 1,536 amplicons across 96 samples within a single plate, with less than 3 hours hands on time.

Customized Design

- Easily create and manage projects using the Illumina online DesignStudio software for a range of amplicon sizes and reference genomes.

Automated Data Analysis

- Perform variant calling and analysis across all samples using simple on-instrument, automated analysis software.

Complete Amplicon Sequencing Solution for MiSeq

- Get the convenience of a fully integrated DNA-to-data solution including online probe design and ordering, assay, sequencing, automated data analysis, and offline software for reviewing results.

DNA Input Recommendations

Type of DNA	Supported Amplicon Size	Input (Up to 15 µl)	A260/A280	FFPE DNA QC
High-quality genomic DNA	150, 175, 250, 425 bp	50 ng (minimum) 250 ng (recommended)	1.8–2.0	Not required
FFPE genomic DNA	150 and 175 bp ONLY	250 ng (minimum)	1.8–2.0	Illumina FFPE QC Kit (WG-321-1001) Δ Cq ≤ 2.0

Input DNA Quantitation

Illumina recommends quantifying the starting genomic material. Quantify the starting genomic material using a fluorescence-based quantification method, such as PicoGreen, rather than a UV-spectrometer-based method. Fluorescence-based methods, which employ a double-stranded DNA (dsDNA) specific dye, specifically and accurately quantitate dsDNA even in the presence of many common contaminants. In contrast, UV spectrometer methods based on 260 OD readings are prone to overestimating DNA concentrations due to the presence of RNA and other contaminants commonly found in gDNA preparations.

Assessing DNA Quality

The Illumina FFPE QC Kit is recommended to determine if FFPE DNA is of sufficient quality for use in with the TruSeq Custom Amplicon Library Preparation. In addition, the ratio of absorbance at 260 nm to absorbance at 280 nm is used as an indication of sample purity. This protocol is optimized for DNA with absorbance ratio values of 1.8–2.0.

Additional Resources

The following resources are available for TruSeq Custom Amplicon Library Preparation protocol guidance and sample tracking. Access these and other resources on the Illumina website at support.illumina.com/sequencing/kits.ilmn. Then, select TruSeq Custom Amplicon Library Preparation **Support**.

Resource	Description
Training	Illustrates elements of the TruSeq Custom Amplicon Library Preparation process. Viewing these videos is strongly recommended for new and less experienced users before starting sample preparation. Click **Training** on **TruSeq Custom Amplicon Library Preparation Support** page.
Best Practices	Provides best practices specific to this protocol. Review before starting sample preparation. Topics include: • General Advice on Sample Handling • Handling Magnetic Beads • Handling Reagents • Avoiding Cross-Contamination Click **Best Practices** on the **TruSeq Custom Amplicon Library Preparation Support** page.
Experienced User Card and Lab Tracking Form (part # 15027985)	Provides protocol instructions, but with less detail than what is provided in this user guide. **New or less experienced users are advised to follow this user guide and not the EUC.** Click **Documentation & Literature** on the **TruSeq Custom Amplicon Library Preparation Support** page.
Illumina Experiment Manager (IEM)	Enables you to create and edit appropriate sample sheets for Illumina sequencers and analysis software and record parameters for your sample plate. To download the software, click **Downloads** on the **TruSeq Custom Amplicon Library Preparation Support** page. To download the documentation, click **Documentation & Literature** on the **TruSeq Custom Amplicon Library Preparation Support** page.

Protocol

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Introduction

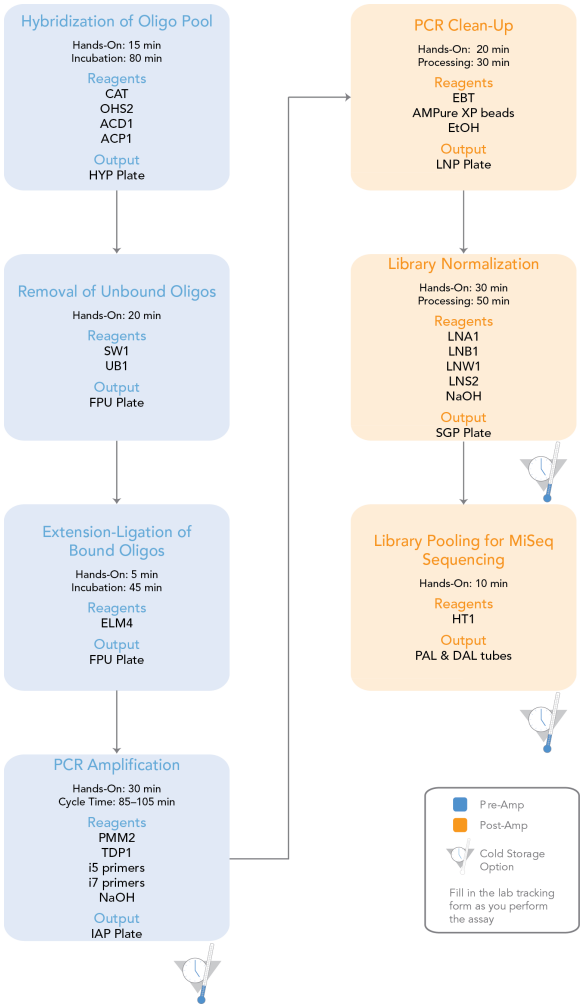
This chapter describes the TruSeq Custom Amplicon protocol.

- Review Best Practices before proceeding. See *Additional Resources* on page 4 for information on how to access TruSeq Custom Amplicon Best Practices on the Illumina website.
- Follow the protocols in the order shown, using the specified volumes and incubation parameters.
- If you are pooling, record information about your samples before beginning library preparation for later use in data analysis.
  - Use IEM to create and edit sample sheets for Illumina sequencers and analysis software. See *Additional Resources* on page 4 for information on how to download IEM software and documentation from the Illumina website.

TruSeq Custom Amplicon Library Preparation Workflow

The following diagram illustrates the workflow using the TruSeq Custom Amplicon Library Preparation Kit. Safe stopping points are marked between steps.

Figure 1 TruSeq Custom Amplicon Library Preparation Workflow



Hybridization of Oligo Pool

During this step, a custom pool containing upstream and downstream oligos specific to your targeted regions of interest is hybridized to your genomic DNA samples.

WARNING

A component in this set of reagents contains formamide, an aliphatic amide that is a probable reproductive toxin. Personal injury can occur through inhalation, ingestion, skin contact, and eye contact. Dispose of containers and any unused contents in accordance with the governmental safety standards for your region.

For more information, see the MSDS for this kit, at www.illumina.com/msds.

Estimated Time

- Total duration: 1 hour 35 minutes
- Hands-on: 15 minutes

Consumables

Item	Quantity	Storage	Supplied By
CAT (Custom Amplicon Oligo Tube)	1 tube	-15°C to -25°C	Illumina
OHS2 (Oligo Hybridization for Sequencing 2)	1 tube	-15°C to -25°C	Illumina
ACD1	1 tube	-15°C to -25°C	Illumina
ACP1	1 tube	-15°C to -25°C	Illumina
Genomic DNA See *DNA Input Recommendations* on page 3.	As needed	-15°C to -25°C	User
96-well skirted PCR plate	1 plate		User
Adhesive aluminum foil seal	2 seals		User
Troughs	As needed		User

Preparation

1. Follow the *DNA Input Recommendations* on page 3 to qualify/quantitate DNA samples.
2. Remove the CAT, OHS2, ACD1, ACP1, and genomic DNA from -15°C to -25°C storage and thaw at room temperature.

   NOTE
   OHS2 might form visible precipitates or crystals. Before use, vortex vigorously, and then hold the tube in front of a light and visually inspect to make sure that all precipitates have dissolved.
3. Set a 96-well heat block to 95°C.
4. Pre-heat an incubator to 37°C to prepare for the extension-ligation step.
5. Create your sample plate layout using the table on the last page of the Experienced User Card or the Illumina Experiment Manager (Select TruSeq Amplicon as the Sample Prep Kit Type). Use the TruSeq Custom Amplicon Calculator for additional guidance. Record the amplicon size, number of amplicons, and the plate positions of each sample DNA/CAT, ACD1/ACP1 (TSCA_Control), and index primers. Illumina recommends processing samples in batches no smaller than 16.

   NOTE
   Illumina requires that you use the provided controls (ACD1/ACP1) in each batch of samples being prepared. Using the provided controls enables Illumina Technical Support to troubleshoot in the event you need assistance. Assistance is not provided unless this control reaction was included in your assay.

   NOTE
   The control ACP1 is specific for Homo sapiens and does not work with DNA from other species.

Procedure

1. Apply the **HYP** (Hybridization Plate) barcode plate sticker to a new 96-well PCR plate.
2. Add 5 µl of control DNA ACD1 and 5 µl of TE or water to 1 well in the **HYP** plate for the assay control.

3 Add 10 µl of Genomic or FFPE DNA to each remaining well of the HYP plate to be used in the assay. For more dilute samples (that is, < 25 ng/µl) up to 15 µl of DNA can be used.

- Example Setup for High Quality Genomic DNA

Input	Volume	DNA Concentration
250 ng	10 µl	25 ng/µl
250 ng	up to 15 µl	≥ 16.7 ng/µl
50 ng	10 µl	5 ng/µl
50 ng	up to 15 µl	≥ 3.3 ng/µl

- Example Setup for FFPE Genomic DNA

Input	Volume	DNA Concentration
250 ng	10 µl	25 ng/µl
250 ng	up to 15 µl	≥ 16.7 ng/µl

4 Add 5 µl of control oligo pool ACP1 to the well containing control DNA ACD1.

5 Using a multichannel pipette, add 5 µl of CAT to the wells containing genomic DNA. Change tips after each column to avoid cross-contamination.

NOTE
To ensure multichannel pipetting accuracy, Illumina recommends pipetting the CAT with a multichannel pipette from a PCR eight-tube strip containing 70 µl of oligo pool in each tube to cover all 12 columns of a plate.

6 If the samples have uneven volumes or are not sitting at the bottom of each well, do the following:

a Seal the **HYP** plate with adhesive aluminum foil, and secure the seal with a rubber roller or sealing wedge.

NOTE
For instructions on viewing a video demonstration of this process, see *Additional Resources* on page 4.

b Centrifuge to 1,000 × g at 20°C for 1 minute.

7 Using a multichannel pipette, add 35 µl of OHS2 to each sample in the **HYP** plate. When dispensing, gently pipette up and down 3–5 times to mix. Change tips after each column to avoid cross-contamination.

NOTE
Ensure any crystals or precipitate in OHS2 have dissolved.

NOTE
Do not mix CAT and OHS2 for storage. If combined, CAT becomes unstable even when stored frozen.

8 Seal the **HYP** plate with adhesive aluminum foil and secure the seal with a rubber roller or sealing wedge.

9 Centrifuge to 1,000 × g at 20°C for 1 minute.

10 Place the **HYP** plate in the pre-heated block at 95°C and incubate for 1 minute.

11 While the plate remains on the pre-heated block, set the temperature to 40°C and continue incubating for 80 minutes.

NOTE
During incubation, the heat block temperature gradually decreases from 95°C to 40°C. This process typically takes 80 minutes. This gradual cooling is critical for proper hybridization; therefore, PCR thermal cyclers with active cooling are not recommended for this process.

Removal of Unbound Oligos

This process removes unbound oligos from genomic DNA using a filter capable of size selection. Two wash steps using SW1 ensure complete removal of unbound oligos. A third wash step using UB1 removes residual SW1 and prepares samples for the extension-ligation step.

WARNING

A component in this set of reagents contains formamide, an aliphatic amide that is a probable reproductive toxin. Personal injury can occur through inhalation, ingestion, skin contact, and eye contact. Dispose of containers and any unused contents in accordance with the governmental safety standards for your region.

For more information, see the MSDS for this kit, at www.illumina.com/msds.

WARNING

This set of reagents contains ß-mercaptoethanol. Perform the following procedure in a hood or well-ventilated area if desired.

Estimated Time

- Total duration: 20 minutes
- Hands-on: 20 minutes

Consumables

Item	Quantity	Storage	Supplied By
ELM4 (thawed in preparation for Extension-Ligation)	1 tube	-15°C to -25°C	Illumina
SW1 (Stringent Wash 1)	1 tube	2°C to 8°C	Illumina
UB1 (Universal Buffer 1)	1 tube	2°C to 8°C	Illumina
Filter plate with lid	1 plate		Illumina
Adapter collar (reusable)	1 plate		Illumina
MIDI plate	1 plate		User
Troughs	As needed		User

Preparation

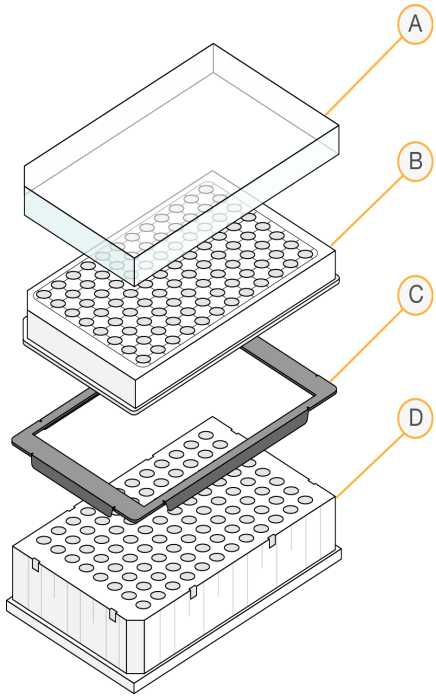
1 Remove ELM4 from -15°C to -25°C storage and thaw at room temperature.
ELM4 is used in the Extension-Ligation step and takes approximately 20 minutes to thaw.

2 Remove SW1 and UB1 from 2°C to 8°C storage and set aside at room temperature.

3 Assemble the filter plate assembly unit (**FPU**) in the following order (from top to bottom):

NOTE
For instructions on viewing a video demonstration of this process, see *Additional Resources* on page 4.

Figure 2 Filter Plate Unit Assembly



A Lid
B Filter plate

C Adapter collar
D MIDI plate

4 Apply the **FPU** barcode plate sticker to the filter plate.

5 Pre-wash the **FPU** plate membrane as follows:
   a Using a multichannel pipette, add 45 µl of SW1 to each well.
   b Cover the **FPU** plate with the filter plate lid and keep it covered during each centrifugation step.
   c Centrifuge the **FPU** at 2,400 × g at 20°C for 10 minutes.

NOTE
Pre-wash only the wells to be used in the current assay. You can use fresh/unused wells of a previously opened filter plate, but do not use wells that have been used in a previous assay.

6 After the pre-wash step, if there is a significant amount (>15 µl/well) of residual buffer in multiple wells (≥10 wells/plate) switch to a fresh filter plate.

NOTE
Illumina strongly recommends keeping spare filter plates (FC-130-1006) on hand as general lab supplies.

Procedure

1 After the 80 minute incubation, confirm that the heat block has cooled to 40˚C. While the **HYP** plate is still in the heat block, reinforce the seal using a rubber roller or sealing wedge.

2 Remove the **HYP** plate from the heat block and centrifuge to 1,000 × g at 20°C for 1 minute to collect condensation.

3 Using a multichannel pipette set to 65 µl, transfer the entire volume of each sample onto the center of the corresponding pre-washed wells of the **FPU** plate. Change tips after each column to avoid cross-contamination.

4 Cover the **FPU** plate with the filter plate lid and centrifuge the **FPU** at 2,400 × g at 20°C for 2 minutes.

5 Wash the **FPU** plate as follows:
   a Using a multichannel pipette, add 45 µl of SW1 to each sample well.
   Changing tips between columns is not required if you use care to avoid cross-contamination.

b Cover the **FPU** plate with the filter plate lid and centrifuge the **FPU** at 2,400 × g for 2 minutes.

NOTE
If the SW1 does not drain completely after 2 minutes, the plate can be centrifuged again for up to 10 minutes. Significantly incomplete drainage of SW1 compromises target enrichment specificity.

6 Repeat the wash as follows:

a Using a multichannel pipette, add 45 µl of SW1 to each sample well.
Changing tips between columns is not required if you use care to avoid cross-contamination.

b Cover the **FPU** plate with the filter plate lid and centrifuge to 2,400 × g for 2 minutes.

NOTE
If the SW1 does not drain completely after 2 minutes, the plate can be centrifuged again for up to 10 minutes. Significantly incomplete drainage of SW1 compromises target enrichment specificity.

7 Discard all the flow-through (containing formamide waste and unbound oligos) collected up to this point in an appropriate hazardous waste container, then reassemble the **FPU**. The same MIDI plate can be reused for the rest of the pre-amplification process.

8 Using a multichannel pipette add 45 µl of UB1 to each sample well.
Changing tips between columns is not required if you use care to avoid cross-contamination.

9 Cover the **FPU** plate with the filter plate lid and centrifuge the **FPU** at 2,400 × g for 2 minutes.

NOTE
If the UB1 does not drain completely after 2 minutes, the plate can be centrifuged again for up to 10 minutes.

Extension-Ligation of Bound Oligos

This process connects the hybridized upstream and downstream oligos. A DNA polymerase extends from the upstream oligo through the targeted region, followed by ligation to the 5′ end of the downstream oligo using a DNA ligase. The extension-ligation results in the formation of products containing the targeted regions of interest flanked by sequences required for amplification.

Estimated Time

- Total duration: 50 minutes
- Hands-on: 5 minutes

Consumables

Item	Quantity	Storage	Supplied By
ELM4 (Extension-Ligation Mix 4)	1 tube	-15°C to -25°C	Illumina
Adhesive aluminum foil seal	1 seal		User
Troughs	As needed		User

Procedure

1. Using a multichannel pipette, add 45 µl of ELM4 to each sample well of the **FPU** plate. The extension-ligation reaction takes place on the filter plate membrane.
   Changing tips between columns is not required if you use care to avoid cross-contamination.
2. Seal the **FPU** plate with adhesive aluminum foil, and then cover with the lid to secure the foil during incubation.
3. Incubate the entire **FPU** assembly in the pre-heated 37°C incubator for 45 minutes.
4. While the **FPU** plate is incubating, prepare the **IAP** (Indexed Amplification Plate) as described in the following section.

PCR Amplification

In this step, the extension-ligation products are amplified using primers that add sample multiplexing index sequences (i5 and i7) as well as common adapters required for cluster generation (P5 and P7).

Estimated Time

- Total duration: 85–105 minutes (depending on the number of PCR cycles used)
- Hands-on: 30 minutes

Consumables

Item	Quantity	Storage	Supplied By
PMM2 (PCR Master Mix 2)	1 tube	-15°C to -25°C	Illumina
i5 primers (A5XX)	1 tube per primer	-15°C to -25°C	Illumina
i7 primers (A7XX)	1 tube per primer	-15°C to -25°C	Illumina
TDP1 (TruSeq DNA Polymerase 1)	1 tube	-15°C to -25°C	Illumina
Microseal 'A' adhesive film	1		User
50 mM NaOH (less than one week old; prepared from 10 N NaOH)	3.5 ml for 96 samples		User
96-well skirted PCR plate	1 plate		User
Troughs	As needed		User

Preparation

1 Record the amplicon size and number of amplicons in your CAT on the Experienced User Card.

This information is used to determine PCR cycling conditions for your assay, and can be found either in DesignStudio or your manifest file.

2 Prepare fresh 50 mM NaOH.

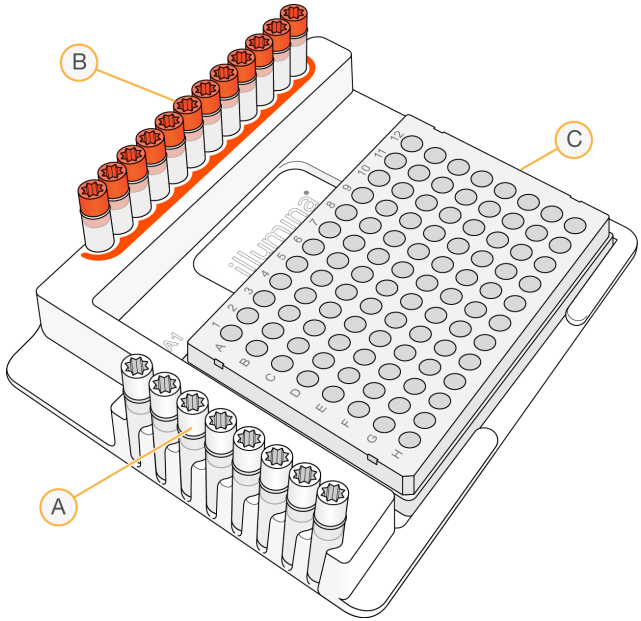
3 Determine the index primers to be used in the assay using the Illumina Experiment Manager and the TruSeq Custom Amplicon Calculator. Record index primer positions on the last page of the Experienced User Card.

4 Remove PMM2 and the index primers (i5 and i7) from -15°C to -25°C storage and thaw on a bench at room temperature.
Allow approximately 20 minutes to thaw PMM2 and the index primers.

5 After the index primers are thawed, vortex each tube to mix and briefly centrifuge the tubes in a microcentrifuge. Use 1.7 ml Eppendorf tubes as adapters for the microcentrifuge.

NOTE
For instructions on viewing a video demonstration of this process, see *Additional Resources* on page 4.

6 Arrange the primers in a rack (i.e. the TruSeq Index Plate Fixture) using the following arrangements:
   a Arrange i5 primer tubes (white caps, clear solution) vertically, aligned with rows A through H.
   b Arrange i7 primer tubes (orange caps, yellow solution) horizontally, aligned with columns 1 through 12.



A i5 primers (white caps)
B i7 primers (orange caps)
C **IAP** plate

7 Apply the **IAP** (Indexed Amplification Plate) barcode plate sticker to a new 96-well PCR plate.

8 Using a multichannel pipette, add 4 µl of i5 primers (clear solution) to each column of the **IAP** plate. Changing tips between columns is not required.

9 To avoid index cross-contamination, discard the original *white* caps and apply new *white* caps provided in the TruSeq Custom Amplicon Index Kit.

10 Using a multichannel pipette, add 4 µl of i7 primers (yellow solution) to each row of the **IAP** plate. ***Tips must be changed after each row to avoid index cross-contamination.***

11 To avoid index cross-contamination, discard the original *orange* caps and apply new *orange* caps provided in the TruSeq Custom Amplicon Index Kit. Remove all the index primer tubes from the working area.

12 For 96 samples, add 56 µl of TDP1 to 2.8 ml of PMM2 (1 full tube). Invert the PMM2/TDP1 PCR master mix 20 times to mix well. You will add this mix to the **IAP** plate in the next section.

NOTE
Always add TDP1 to PMM2 before use. Never store the combined PMM2/TDP1 master mix.

Procedure

1 When the 45 minute extension-ligation reaction is complete, remove the **FPU** from the incubator. Remove the aluminum foil seal and replace with the filter plate lid. Removing the aluminum foil seal before centrifugation is recommended to ensure the reaction supernatant drains into the waste plate effectively.

2 Centrifuge the **FPU** at 2,400 × g for 2 minutes.

3 Using a multichannel pipette, add 25 µl of 50 mM NaOH to each sample well on the **FPU** plate. Ensuring that pipette tips come in contact with the membrane, pipette the NaOH up and down 5–6 times. Tips must be changed after each column.

4 Incubate the **FPU** plate at room temperature for 5 minutes.

5 While the **FPU** plate is incubating, use a multichannel pipette to transfer 22 µl of the PMM2/TDP1 PCR master mix to each well of the **IAP** plate containing index primers. Change tips between samples.

6 Transfer samples eluted from the **FPU** plate to the **IAP** plate as follows:

a Set a multichannel P20 pipette to 20 µl.

b Using fine tips, pipette the NaOH in the first column of the **FPU** plate up and down 5–6 times. Then transfer 20 µl from the **FPU** plate to the corresponding column of the **IAP** plate. Gently pipette up and down 5–6 times to combine the DNA with the PCR master mix.

NOTE
Slightly tilt the **FPU** plate to ensure complete aspiration and to avoid air bubbles.

c Transfer the remaining columns from the **FPU** plate to the **IAP** plate in a similar manner. Tips must be changed after each column to avoid index and sample cross-contamination.

d After all the samples have been transferred, the waste collection MIDI plate of the **FPU** can be discarded. Put the metal adapter collar away for future use. If only a

partial **FPU** plate is used, clearly mark which wells have been used. Store the **FPU** plate and lid in a sealed plastic bag to avoid contamination of the filter membrane.

7 Cover the **IAP** plate with Microseal 'A' film and seal with a rubber roller.

8 Centrifuge to 1,000 × g at 20°C for 1 minute.

9 Transfer the **IAP** plate to the post-amplification area.

10 Perform PCR on a thermal cycler using the following program and the recommended number (X) of PCR cycles. The following tables contain amplicon size, number of amplicons in your CAT, type of DNA input, and DNA input quantity to help you calculate the number of PCR cycles required.

NOTE
The ACD1/ACP1 control can be processed using the same conditions as your CAT.

Table 1 100–250 ng

Amplicon Size	150/175 bp		250 bp	425 bp
DNA Input	High Quality	FFPE	High Quality	High Quality
	Number of PCR Cycles (X)			
<96 amplicons	29	31	30	30
97–384 amplicons	25	27	25	26
385–768 amplicons	23	25	24	25
769–1,536 amplicons	22	24	23	24

Table 2 50–99 ng

Amplicon Size	150/175 bp	250 bp	425 bp
DNA Input	High Quality	High Quality	High Quality
	Number of PCR Cycles (X)		
<96 amplicons	32	33	33
97–384 amplicons	28	28	29
385–768 amplicons	26	27	28
769–1,536 amplicons	25	26	27

- 95°C for 3 minutes
- X cycles of:
  - 95°C for 30 seconds
  - 66°C for 30 seconds
  - 72°C for 60 seconds
- 72°C for 5 minutes
- Hold at 10°C

SAFE STOPPING POINT

If you do not plan to proceed to *PCR Clean-Up on page 23* immediately, the plate can remain on the thermal cycler overnight. You can also store it at 2°C to 8°C up to two days. If storing at 2°C to 8°C, replace Microseal 'A' with Microseal 'B'

PCR Clean-Up

This process uses AMPure XP beads to purify the PCR products from the other reaction components.

Estimated Time

- Total duration: 50 minutes
- Hands-on: 20 minutes

Consumables

Item	Quantity	Storage	Supplied By
EBT (Elution Buffer with Tris)	1 tube	Room temperature	Illumina
AMPure XP beads	As needed	2°C to 8°C	User
Freshly Prepared 80% Ethanol (EtOH)	40 ml per 96 samples	Room temperature	User
96-well MIDI plates	2		User
Microseal 'B' adhesive film	As needed		User
Troughs	As needed		User

Preparation

1. Bring the AMPure XP beads to room temperature.
2. Prepare fresh 80% ethanol from absolute ethanol.

NOTE
Always prepare fresh 80% ethanol for wash steps. Ethanol can absorb water from the air impacting your results.

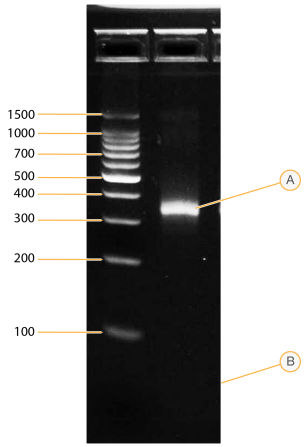
Procedure

1. Centrifuge the **IAP** plate at 1,000 × g at 20°C for 1 minute to collect condensation.

2 To confirm that the library successfully amplified, run an aliquot of the control and selected test samples on a 4% agarose (5 µl) or on a Bioanalyzer (1 µl). The expected PCR product sizes for each amplicon length are indicated in the following table.

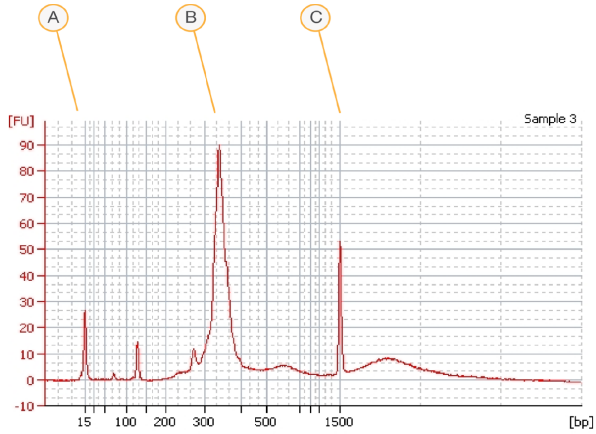
Amplicon Size	Expected PCR Product Size
150 bp	~280 bp
175 bp	~310 bp
250 bp	~350 bp
425 bp	~570 bp

Figure 3 Agarose Gel Example (Expected ACP1/ACD1 PCR product is shown)



A Expected PCR Product for 250 bp amplicons (~350 bp)
B Primers

Figure 4 Bioanalyzer Example (Expected ACP1/ACD1 PCR product is shown)



A Marker
B Expected PCR Product for 250 bp amplicons (~350bp)
C Marker

NOTE

Performing assessment of library quality by gel electrophoresis or Bioanalyzer is highly recommended for TruSeq CAT oligo pools, which are being used for the first time. It is not necessary to perform this assessment on every sample in the experiment.

Illumina requires that you also include the control reaction generated with ACD1 and ACP1 in this assessment to enable Illumina Technical Support to troubleshoot in the event you need assistance.

3 Apply the **CLP** (Clean-up Plate) barcode plate sticker to a new MIDI plate.

4 Using a multichannel pipette, add the appropriate volume of AMPure XP beads indicated in the following table, corresponding to your amplicon size, to each well of the **CLP** plate.

NOTE
The ACD1/ACP1 control can be processed using the same conditions as your CAT.

Amplicon Size	µl AMPure XP beads
150 bp	60 µl
175 bp	60 µl
250 bp	45 µl
425 bp	35 µl

5 Using a multichannel pipette set to 60 µl, transfer the entire PCR product from the **IAP** plate to the **CLP** plate. Change tips between samples.

NOTE
For instructions on viewing a video demonstration of this process, see *Additional Resources* on page 4.

6 Seal the **CLP** plate with a Microseal 'B' adhesive seal.

7 Shake the **CLP** plate on a microplate shaker at 1,800 rpm for 2 minutes.

8 Incubate at room temperature without shaking for 10 minutes.

9 Place the plate on a magnetic stand for 2 minutes or until the supernatant has cleared.

10 With the **CLP** plate on the magnetic stand and a multichannel pipette set to 100 µl, carefully remove and discard the supernatant. Change tips between samples.

NOTE
If any beads are aspirated into the tips, dispense the beads back to the plate and let the plate rest on the magnet for 2 minutes, and then make sure that the supernatant is clear.

11 With the **CLP** plate on the magnetic stand, wash the beads with freshly prepared 80% ethanol as follows:

a Using a multichannel pipette, add 200 µl of freshly prepared 80% ethanol to each sample well. Changing tips is not required if you use care to avoid cross-contamination. You do not need to resuspend the beads currently.
b Incubate the plate on the magnetic stand for 30 seconds or until the supernatant appears clear.
c Carefully remove and discard the supernatant.

12 With the **CLP** plate on the magnetic stand, perform a second ethanol wash as follows:
a Using a multichannel pipette, add 200 µl of freshly prepared 80% ethanol to each sample well.
b Incubate the plate on the magnetic stand for 30 seconds or until the supernatant appears clear.
c Carefully remove and discard the supernatant.
d Use a P20 multichannel pipette to remove excess ethanol.

13 Remove the **CLP** plate from the magnetic stand and allow the beads to air-dry for 10 minutes.

14 Using a multichannel pipette, add 30 µl of EBT to each well of the **CLP** plate. Changing tips is not required if you use care to avoid cross-contamination.

15 Seal the plate with a Microseal 'B' adhesive seal.

16 Shake the **CLP** plate on a microplate shaker at 1,800 rpm for 2 minutes.

NOTE
Ensure that all samples are resuspended. If there are samples in which the beads are not resuspended, gently pipette up and down to resuspend the beads and repeat the previous two steps.

17 Incubate at room temperature without shaking for 2 minutes.

18 Place the plate on the magnetic stand for 2 minutes or until the supernatant has cleared.

19 Apply the **LNP** (Library Normalization Plate) barcode plate sticker to a new MIDI plate.

20 Using a P20 multichannel pipette and fine tips, carefully transfer 20 µl of the supernatant from the **CLP** plate to the **LNP** plate. Change tips between samples to avoid cross-contamination.

NOTE
If any beads are aspirated into the tips, dispense the beads back to the plate and let the plate rest on the magnet for 2 minutes, and then make sure that the supernatant is clear.

21 Seal the **LNP** plate with Microseal 'B' and then centrifuge to 1,000 × g for 1 minute to ensure all the supernatant is at the bottom of the well.

Library Normalization

This process normalizes the quantity of each library to ensure more equal library representation in your pooled sample.

Estimated Time

- Total duration: 1 hour 20 minutes
- Hands-on: 30 minutes

Consumables

Item	Quantity	Storage	Supplied By
LNA1 (Library Normalization Additives 1)	1 tube	-15°C to -25°C	Illumina
LNB1 (Library Normalization Beads 1)	1 tube	2°C to 8°C	Illumina
LNW1 (Library Normalization Wash 1)	2 tubes	2°C to 8°C	Illumina
LNS2 (Library Normalization Storage buffer 2)	1 tube	Room temperature	Illumina
0.1 N NaOH (less than one week old)	3 ml per 96 samples		User
96-well skirted PCR plate	1 plate		User
15 ml conical tube	1 tube		User
Microseal 'B' adhesive film	As needed		User

WARNING

A component in this set of reagents contains formamide, an aliphatic amide that is a probable reproductive toxin. Personal injury can occur through inhalation, ingestion, skin contact, and eye contact. Dispose of containers and any unused contents in accordance with the governmental safety standards for your region.

For more information, see the MSDS for this kit, at www.illumina.com/msds.

WARNING
This set of reagents contains ß-mercaptoethanol. Perform the following procedure in a hood or well-ventilated area if desired.

Preparation

NOTE
For instructions on viewing a video demonstration of this process, see *Additional Resources* on page 4.

1 Prepare fresh 0.1N NaOH.

2 Remove LNA1 from -15°C to -25°C storage and bring to room temperature. Use a 20°C to 25°C water bath as needed.

NOTE
LNA1 might form visible precipitates or crystals. Before use, vortex vigorously, and then hold the tube in front of a light and visually inspect to make sure that all precipitate has dissolved.

3 Remove LNB1 and LNW1 from 2°C to 8°C storage and bring to room temperature. Use a 20°C to 25°C water bath as needed.

4 Vigorously vortex LNB1 for at least 1 minute with intermittent inversion until the beads are well-resuspended and no pellet is found at the bottom of the tube when the tube is inverted.

Procedure

1 For 96 samples, add 4.4 ml of LNA1 to a fresh 15 ml conical tube.

2 Use a P1000 pipette set to 1000 µl to resuspend LNB1 thoroughly by pipetting up and down 15–20 times, until the bead pellet at the bottom is resuspended.

NOTE
It is critical to completely resuspend the LNB1 bead pellet at the bottom of the tube. The use of a P1000 ensures that the beads are homogeneously resuspended and that there is no bead mass at the bottom of the tube. Resuspension is essential for achieving consistent cluster density on the flow cell.

3 Immediately after LNB1 is thoroughly resuspended, use a P1000 pipette to transfer 800 µl of LNB1 to the 15 ml conical tube containing LNA1. Mix well by inverting the

tube 15–20 times. The resulting LNA1/LNB1 bead mix is enough for 96 samples. Pour the bead mix into a trough and use it immediately in the next step.

NOTE
If you do not plan to use full tubes for 96 samples, a P1000 set to 1000 µl is required to resuspend the beads completely in step 2. Mix only the required amounts of LNA1 and LNB1 for the current experiment. Never use a P200 pipette to handle LNB1. Store the remaining LNA1 and LNB1 separately at their respective recommended temperatures. To preserve stability, never freeze LNB1 beads or mix with LNA1 if not used immediately.

4 Using a multichannel pipette, add 45 µl of the combined LNA1/LNB1 to each well of the **LNP** plate containing libraries. Changing tips between columns is not required if you use care to avoid cross-contamination.

5 Seal the **LNP** plate with a Microseal 'B' adhesive seal.

6 Shake the **LNP** plate on a microplate shaker at 1,800 rpm for 30 minutes.

NOTE
The 30 minute incubation is critical for proper library normalization. Incubations of greater or less than 30 minutes affect library representation and cluster density.

7 Place the plate on a magnetic stand for 2 minutes or until the supernatant has cleared.

8 With the **LNP** plate on the magnetic stand, use a multichannel pipette set to 80 µl to remove the supernatant and then discard in an appropriate hazardous waste container.

NOTE
If any beads are inadvertently aspirated into the tips, dispense the beads back to the plate and let the plate rest for 2 minutes or until the supernatant has cleared.

9 Remove the **LNP** plate from the magnetic stand and wash the beads with LNW1, as follows:

a Using a multichannel pipette, add 45 µl of LNW1 to each sample well. Changing tips between columns is not required if you use care to avoid cross-contamination.

b Seal the **LNP** plate with a Microseal 'B' adhesive seal.

c Shake the **LNP** plate on a microplate shaker at 1,800 rpm for 5 minutes.

d Place the plate on the magnetic stand for 2 minutes or until the supernatant has cleared.

e Carefully remove and discard the supernatant in an appropriate hazardous waste container.

10 Remove the **LNP** plate from the magnetic stand and repeat the wash with LNW1, as follows:

a Using a multichannel pipette, add 45 µl of LNW1 to each well.
Changing tips between columns is not required if you use care to avoid cross-contamination.

b Seal the **LNP** plate with a Microseal 'B' adhesive seal.

c Shake the **LNP** plate on a microplate shaker at 1,800 rpm for 5 minutes.

d Place the plate on the magnetic stand for 2 minutes or until the supernatant has cleared.

e Carefully remove and discard the supernatant in an appropriate hazardous waste container.

f Use a P20 multichannel pipette to remove excess LNW1.

NOTE
Using a P20 multichannel to remove residual LNW1 is important to avoid reagent carryover into the storage buffer, and to reduce volume variability, which would affect library normalization.

11 Remove the **LNP** plate from the magnetic stand and add 30 µl of 0.1 N NaOH (less than a week old) to each well to elute the sample.

12 Seal the **LNP** plate with a Microseal 'B' adhesive seal.

13 Shake the **LNP** plate on a microplate shaker at 1,800 rpm for 5 minutes.

14 During the 5 minute elution, apply the **SGP** (Storage Plate) barcode plate sticker to a new 96-well PCR plate.

15 Add 30 µl LNS2 to each well to be used in the **SGP** plate.

16 After the 5 minute elution, make sure that all samples in the **LNP** plate are resuspended completely. If the samples are not resuspended, gently pipette up and down or lightly tap the plate on the bench to resuspend the beads. Then shake for another 5 minutes.

17 Place the **LNP** plate on the magnetic stand for 2 minutes or until the liquid is clear.

18 Using a multichannel pipette set to 30 µl, transfer the supernatant from the **LNP** plate to the **SGP** plate. Change tips between samples to avoid cross-contamination.

NOTE

If any beads are aspirated into the tips, dispense the beads back to the plate and let the plate rest on the magnet for 2 minutes, and then make sure that the supernatant is clear.

19 Seal the **SGP** plate with Microseal 'B' and then centrifuge to 1,000 × g for 1 minute.

NOTE

The final library pool consists of single-stranded DNA, which does not resolve well on an agarose gel or Bioanalyzer chip. qPCR can be used for quality control if desired. For more information, please see the *Sequencing Library qPCR Quantification Guide*.

SAFE STOPPING POINT

If you do not plan to proceed to *Library Pooling and MiSeq Sample Loading* and subsequent sequencing on the MiSeq, store the sealed **SGP** plate at -15°C to -25°C.

Library Pooling and MiSeq Sample Loading

In preparation for cluster generation and sequencing, equal volumes of normalized library are combined, diluted in hybridization buffer, and heat denatured before sequencing on the MiSeq.

Estimated Time

- Total duration: 10 minutes
- Hands-on: 10 minutes

Consumables

Item	Quantity	Storage	Supplied By
HT1 (Hybridization buffer)	1 tube	-15°C to -25°C	Illumina
Eppendorf tubes (screw-cap recommended)	2 tubes		User
PCR eight-tube strip	1		User
2.5 L Ice bucket	1		User

Preparation

1. Set a heat block suitable for 1.5 ml centrifuge tubes to 96°C.
2. Remove a MiSeq reagent cartridge from -15°C to -25°C storage and thaw at room temperature.
3. In an ice bucket, prepare an ice-water bath by combining 3 parts ice and 1 part water.

Procedure

1. If the **SGP** plate was stored frozen, thaw the **SGP** plate at room temperature.
2. Centrifuge the **SGP** plate at 1,000 × g at 20°C for 1 minute to collect condensation.
3. Apply the **PAL** (Pooled Amplicon Library) barcode sticker to a fresh Eppendorf tube.

4 Determine the samples to be pooled for sequencing. Calculate your supported sample multiplexing level, based on the number of targeted regions and desired coverage. Use the following table or the TruSeq Custom Amplicon Calculator (on the TruSeq Custom Amplicon Library Preparation support page on the Illumina website).

Amplicons per CAT	Desired Mean Coverage*	Suggested Maximum Samples per MiSeq Run*	
		MiSeq v2	MiSeq v3
16	150x	96	96
	500x	96	96
48	150x	96	96
	500x	96	96
96	150x	96	96
	500x	96	96
384	150x	96	96
	500x	48	80
768	150x	72	96
	500x	24	40
1536	150x	36	60
	500x	12	20

* Actual performance varies depending on the genes being targeted. If unexpectedly low coverage is found with certain genes in a newly designed CAT, deeper sequencing might be required.

5 If the **SGP** plate was stored frozen, mix each library to be sequenced by pipetting up and down 3–5 times using a P200 multichannel pipette. Change tips between samples.

6 Using a P20 multichannel pipette, transfer 5 µl of each library to be sequenced from the **SGP** plate, column by column, to a PCR eight-tube strip. Change tips after each column to avoid cross-contamination. Seal **SGP** with Microseal 'B' and set aside.

NOTE

For instructions on viewing a video demonstration of this process, see *Additional Resources* on page 4.

NOTE
After use, store the sealed **SGP** plate at -15°C to -25°C.

7 Combine and transfer the contents of the PCR eight-tube strip into the **PAL** tube. Mix **PAL** well.

8 Apply the **DAL** (Diluted Amplicon Library) barcode sticker to a fresh Eppendorf tube.

9 Create **DAL** by combining the volumes of HT1 and **PAL** indicated in Table 3 based on your MiSeq Reagent Kit version. Upon transferring PAL, using the same tip, pipette up and down 3–5 time to rinse the tip and ensure complete transfer.

NOTE
Volumes for diluting PAL with HT1 were established using recommended equipment (e.g., plate shaker calibrated for shaking speed). Typical laboratory conditions (e.g., 20°C to 25°C) were strictly followed during the normalization procedure. If cluster density is too high or too low, adjust the dilution ratio to better suit the equipment, temperature, and handling in your laboratory after validation.

Table 3 Pooling Dilution

	Volume of HT1	Volume of PAL
MiSeq v2	594 µl	6 µl
MiSeq v3	580 µl	20 µl

10 Mix **DAL** by vortexing the tube at top speed.

NOTE
If you would like to save the remaining **PAL** for future use, store the **PAL** tube at -15°C to -25°C.

Make sure that the diluted library **DAL** is freshly prepared and used immediately for MiSeq loading. Storage of the **DAL** results in a significant reduction of cluster density.

11 Using a heat block, incubate the **DAL** tube at 96°C for 2 minutes.

12 After the incubation, invert **DAL** 1–2 times to mix and immediately place in the ice-water bath.

13 Keep the **DAL** tube in the ice-water bath for 5 minutes.

NOTE
Perform the heat denaturation step immediately before loading **DAL** into the MiSeq reagent cartridge to ensure efficient template loading on the MiSeq flow cell.

14 Load **DAL** into a thawed MiSeq reagent cartridge into the **Load Samples** reservoir.

15 Sequence your library as indicated in the *MiSeq System User Guide*.

NOTE
Illumina recommends choosing a read length that does not exceed the CAT amplicon size.

Supporting Information

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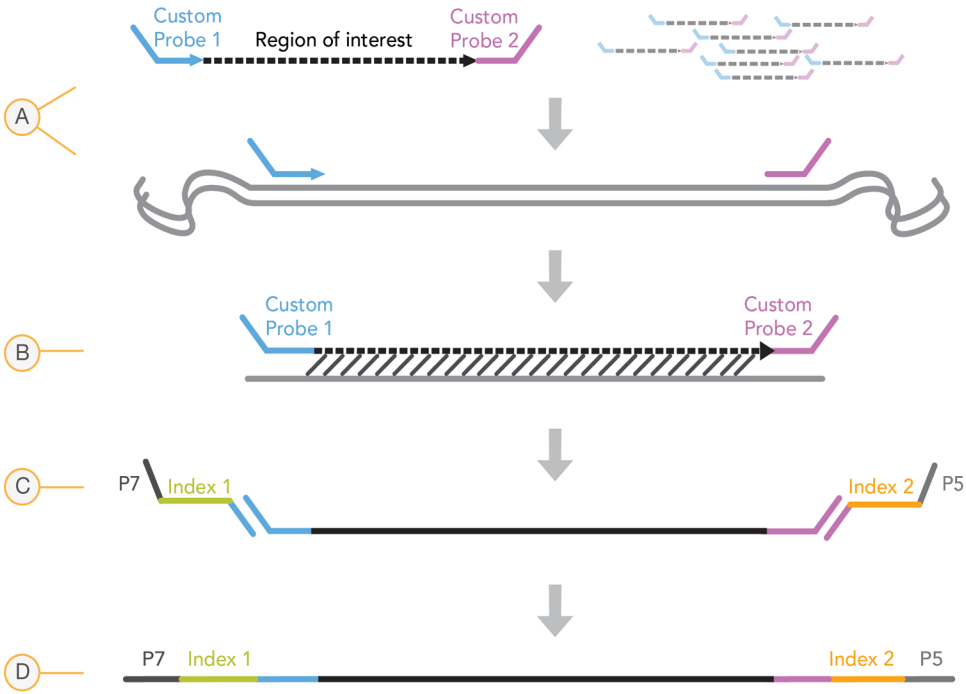
Introduction

The protocols described in this guide assume that you have reviewed the contents of this appendix, confirmed your kit contents, and obtained all of the requisite consumables and equipment.

How Does the TruSeq Custom Amplicon Library Preparation Assay Work?

One pair of oligos is designed for each amplicon. Hybridization of oligos to genomic DNA occurs in a 96-well plate, followed by extension and ligation to form DNA templates consisting of the regions of interest flanked by universal primer sequences. Using indexed primers supplied with the kit, DNA templates are amplified by PCR, pooled into a single tube, and sequenced on the MiSeq System.



A Hybridization of custom oligonucleotide probes
B Extension and ligation
C Addition of indices and sequencing adapters by PCR
D Final amplicon ready for sequencing with MiSeq

TruSeq Custom Amplicon Library Preparation Process Overview

The TruSeq Custom Amplicon Library Preparation process overview can be summarized into the following steps:

Design Your Project

To design the oligos for targeting your regions of interest, use the Illumina DesignStudio. DesignStudio is a free, simple interface for custom probe design, providing dynamic feedback to select optimal amplicons and estimates of total project pricing. You can access DesignStudio through your MyIllumina account. Follow the links in the **Custom Products** tab to access DesignStudio for TruSeq Custom Amplicon.

Place Your Order

When you have completed your design through DesignStudio, place your order directly through your MyIllumina account. You can access the **TruSeq Custom Amplicon Calculator**, a convenient tool to help you calculate your supported sample multiplexing level based on the number of targeted regions and desired coverage. Go to the TruSeq Custom Amplicon Library Preparation support page on the Illumina website. A MyIllumina account is required. When placing your order, make sure that you also order MiSeq reagents and additional components needed to perform the TSCA Sample Preparation. See the *TruSeq Custom Amplicon Library Preparation Kit Contents* on page 46 in this guide for information on additional components such as index primers.

Download Your Manifest File

The manifest file is required for TruSeq Custom Amplicon sequencing on the MiSeq. It provides the list of regions targeted in your project, which MiSeq uses for alignment and analysis. The manifest file can be downloaded when your order ships. You can access all your manifest files for current or previous projects through your MyIllumina account. Follow the links in the **Custom Products** tab to access the Product Files for TruSeq Custom Amplicon.

Prepare Libraries and Sample Sheet

Prepare your libraries using the protocol detailed in this user guide. In parallel, prepare a sample sheet, which the MiSeq uses to identify each sample and its corresponding index.

To prepare your sample sheet, use the Illumina Experiment Manager, a wizard-based application that allows the recording of your sample ID, workflow, indices, and other parameters applicable to your 96-well plate. The Illumina Experiment Manager can be run on any Windows platform. You can download the Illumina Experiment Manager from the Illumina website at www.illumina.com. Go to the TruSeq Custom Amplicon Library Preparation support page and click **Downloads**. A MyIllumina account is required.

Sequence Libraries on MiSeq

TruSeq Custom Amplicon Libraries must be sequenced on a MiSeq sequencing system using a paired-end sequencing run. For more details on using the MiSeq instrument or setting up your run, see the *MiSeq System User Guide*.

Analyze Data with Illumina Supported Software

Analyze Data with MiSeq Reporter — MiSeq Reporter processes the base calls generated by the MiSeq sequencing system. It is an on-instrument software, which is built in to the instrument's processes. MiSeq Reporter produces information such as alignment and structural variants. For TruSeq Custom Amplicon libraries, it produces aligned reads in the BAM format and outputs variant calls in .vcf files. For more information about this software, see the *MiSeq System User Guide* or MiSeq Reporter's online help (www.illumina.com/help/miseq_reporter/default.htm).

Illumina Amplicon Viewer — The Illumina Amplicon Viewer has been designed and developed for off-instrument visualization and analysis of TruSeq Custom Amplicon data. Amplicon Viewer allows you to view data (including coverage, Q-score, variant calls, etc.) from multiple MiSeq amplicon runs simultaneously and interactively. DesignStudio might need to cover a large contiguous region with multiple amplicons, and the Illumina Amplicon Viewer opens the reconstituted contiguous region with combined coverage and variants appropriately. You can also export custom reports based on selected samples/targets/variants. You can download the Amplicon Viewer through your MyIllumina account. For more information about this software, see www.illumina.com/help/amplicon_viewer/default.htm.

Acronyms

Table 4 TruSeq Custom Amplicon Library Preparation Acronyms

Acronym	Definition
ACD1	Amplicon Control DNA 1
ACP1	Amplicon Control Oligo Pool 1
CAT	Custom Amplicon Oligo Tube
CLP	CLean-up Plate
DAL	Diluted Amplicon Library
EBT	Elution Buffer with Tris
ELM4	Extension Ligation Mix 4
FPU	Filter Plate Unit
HT1	Hybridization Buffer
HYP	Hybridization Plate
IAP	Indexed Amplification Plate
LNA1	Library Normalization Additives 1
LNB1	Library Normalization Beads 1
LNP	Library Normalization Plate
LNS2	Library Normalization Storage Buffer 2
LNW1	Library Normalization Wash 1
OHS2	Oligo Hybridization for Sequencing Reagent 2
PAL	Pooled Amplicon Library

Acronym	Definition
PMM2	PCR Master Mix 2
SGP	StoraGe Plate
SW1	Stringent Wash 1
TDP1	TruSeq DNA Polymerase 1
UB1	Universal Buffer 1

TruSeq Custom Amplicon Library Preparation Kit Contents

The TruSeq Custom Amplicon Library Preparation Kit contains the following components and is shipped on dry ice unless specified otherwise. As soon as you receive your kit, store the kit components at the specified temperatures and in designated pre-amplification and post-amplification areas.

TruSeq Custom Amplicon Library Preparation Kit v1.5 (Catalog # FC-130-1001)

- Box 1, Pre-Amplification

Acronym	Reagent Name	Storage Temperature	Area
ACD1	Amplicon Control DNA 1	-15°C to -25°C	Pre-Amp
ACP1	Amplicon Control Oligo Pool 1	-15°C to -25°C	Pre-Amp
OHS2	Oligo Hybridization for Sequencing Reagent 2	-15°C to -25°C	Pre-Amp
ELM4	Extension Ligation Mix 4	-15°C to -25°C	Pre-Amp
PMM2	PCR Master Mix 2	-15°C to -25°C	Pre-Amp
TDP1	TruSeq DNA Polymerase 1	-15°C to -25°C	Pre-Amp
SW1	Stringent Wash 1	2°C to 8°C	Pre-Amp
UB1	Universal Buffer 1	2°C to 8°C	Pre-Amp
	Barcode plate stickers for HYP, FPU, IAP	Room temperature	Pre-Amp

WARNING

A component in this set of reagents contains formamide, an aliphatic amide that is a probable reproductive toxin. Personal injury can occur through inhalation, ingestion, skin contact, and eye contact. Dispose of containers and any unused contents in accordance with the governmental safety standards for your region.

For more information, see the MSDS for this kit, at www.illumina.com/msds.

- Box 2, Pre-Amplification

This box is shipped at room temperature. As soon as you receive your kit, **remove LNB1 from box 2 and store at 2°C to 8°C in the post-amplification area. Keep the filter plate in the pre-amplification area at room temperature**.

Acronym	Reagent Name	Storage Temperature	Area
	Filter plate with lid	Room temperature	Pre-Amp
LNB1	Library Normalization Beads 1	2°C to 8°C	Post-Amp

- Box 3, Post-Amplification

Acronym	Reagent Name	Storage Temperature	Area
HT1	Hybridization Buffer	-15°C to -25°C	Post-Amp
LNA1	Library Normalization Additives 1	-15°C to -25°C	Post-Amp
LNW1	Library Normalization Wash 1	2°C to 8°C	Post-Amp
LNS2	Library Normalization Storage Buffer 2	Room temperature	Post-Amp
EBT	Elution Buffer with Tris	Room temperature	Post-Amp
	Barcode plate stickers for CLP, LNP, SGP, PAL, DAL	Room temperature	Post-Amp

- Box 4, TruSeq Custom Amplicon Oligo Kit, Pre-Amplification

Acronym	Reagent Name	Storage Temperature	Area
CAT	Custom Amplicon oligo Tube	-15°C to -25°C	Pre-Amp

TruSeq Custom Amplicon Library Preparation Index Kit (Catalog # FC-130-1003)

- Box 1, Pre-Amplification

Reagent Name	Storage Temperature	Area
i5 Index Primers, A501 to A508 (8 tubes)	-15°C to -25°C	Pre-Amp
i7 Index Primers, A701 to A712 (12 tubes)	-15°C to -25°C	Pre-Amp

- Box 2, Pre-Amplification

Reagent Name	Storage Temperature	Area
i5 Index Tube Caps, White	Room temperature	Pre-Amp
i7 Index Tube Caps, Orange	Room temperature	Pre-Amp

Additional Components

Consumable	Catalog #	Storage Temperature	Area
TruSeq Index Plate Fixture Kit (Required and reusable)	FC-130-1005	Room temperature	Pre-Amp
TruSeq Custom Amplicon Filter Plate (Highly recommended)	FC-130-1006	Room temperature	Pre-Amp
TruSeq Index Plate Fixture and Collar Kit (Required and reusable)	FC-130-1007	Room temperature	Pre-Amp
Illumina FFPE QC Kit (Highly recommended for FFPE DNA samples)	WG-321-1001	-15°C to -25°C	Pre-Amp

User-Supplied Consumables

Quantity	Consumable	Supplier
As needed	10 N NaOH (prepare from tablets or use a standard solution)	General lab supplier
3	96-well skirted PCR plates, 0.2 ml, polypropylene	Bio-Rad, Part # MSP-9601
3	96-well storage plates, 0.8 ml (MIDI plates)	Fisher Scientific, Part # AB-0859 Fisher Scientific, Part # AB-0765
As needed	Agencourt AMPure XP, 60 ml kit	Beckman Coulter, Part # A63881/A63880
3	Adhesive aluminum foil seal	Beckman Coulter, Part # 538619
As needed	Conical tubes, 15 ml	General lab supplier
2	Eppendorf microcentrifuge tubes (screw top recommended)	General lab supplier
40 ml	Ethanol, 200 proof for molecular biology	General lab supplier
1	Microseal 'A' adhesive seals	Bio-Rad, Part # MSA-5001
As needed	Microseal 'B' adhesive seals	Bio-Rad, Part # MSB-1001
2	PCR Eight-Tube Strips	General lab supplier
As needed	Solution basin, PVC, non-sterile (trough)	Labcor, Part# 730-001
As needed	Agarose gel (2% for 250- and 425-bp amplicons, or 4% for 150-, 175-, and 250-bp amplicons)	General Lab Supplier
As needed	DNA 1000 Kit for Bioanalyzer	Agilent 5067-1504 (for 300 samples)
As needed	DNA molecular weight markers	General Lab Supplier
As needed	Ice bucket	General Lab Supplier

Equipment

Pre-PCR

Equipment	Supplier
37° Incubator	Forced Air Oven, VWR International or comparable
Heat Block, 96-well	Scigene, Hybex Microsample Incubator for PCR plate Note: This model is recommended for this assay. Passive cooling, as opposed to active cooling performed in a PCR thermocycler, is recommended for maximum target enrichment specificity and uniformity.
Tabletop Centrifuge	General lab supplier (Plate centrifuge that attains designated speeds of protocol)

NOTE
Use a dedicated set of pipettes, pipette tips, vortexer, and centrifuge for pre-amplification steps.

Post-PCR

Equipment	Supplier
Magnetic Stand-96	Invitrogen DynaMag™-96 Side Skirted
Post-PCR Plate Shaker	Q Instruments, BioShake iQ High Speed Thermal mixer, Part#1808-0506 or Q Instruments, BioShake XP High Speed mixer, Part#1808-0505
Tabletop Centrifuge	General lab supplier (Plate centrifuge that attains designated speeds of protocol)
Gel Electrophoresis Supplies and Apparatus	General lab supplier
[Optional] Bioanalyzer System	Agilent Technologies
Heat Block for 1.5 ml centrifuge tubes	General lab supplier

NOTE
Use a dedicated set of pipettes, pipette tips, vortexer, heat block, and centrifuge for post-amplification steps.

Thermal Cycler

The following table lists the recommended settings for selected thermal cycler models. Illumina recommends that you validate any thermal cyclers not listed if your lab has not yet performed the TruSeq Custom Amplicon Library Preparation protocol.

Thermal Cycler	Temp Mode	Lid Temp	Vessel Type
Bio-Rad DNA Engine Tetrad 2	Calculated	Heated, Constant at 100°C	Polypropylene plates and tubes
MJ Research DNA Engine Tetrad	Calculated	Heated	Plate
Eppendorf Mastercycler Pro S	Gradient S, Simulated Tube	Heated	Plate

MiSeq Sample Sheet Preparation

Create your Sample Sheet for MiSeq sequencing according to the *MiSeq Sample Sheet Quick Reference Guide*. Illumina recommends the Illumina Experiment Manager to prepare your Sample Plate and Sample Sheet. Select MiSeq as your instrument, then select Targeted Resequencing and TruSeq Amplicon as the workflow. Make sure that you select the genome folder appropriate for the species used to design your CAT. Alternatively, you can use your Experienced User Card and Lab Tracking Form and the appropriate index sequences, corresponding to the PCR primers used in your assay.

NOTE
Give the assay control prepared with ACD1/ACP1 the Sample ID and Sample Name "TSCA_Control" in your Sample Plate and Sample Sheet files.

NOTE
If you have used FFPE samples as your DNA input, make sure that the "Use Somatic Variant Caller" box is checked under TruSeq Amplicon Workflow-Specific Settings.

NOTE
Illumina recommends choosing a read length that does not exceed the CAT amplicon size.

i7 Index PCR Primer	Index Sequence
A701	ATCACGAC
A702	ACAGTGGT
A703	CAGATCCA
A704	ACAAACGG
A705	ACCCAGCA
A706	AACCCCTC
A707	CCCAACCT
A708	CACCACAC
A709	GAAACCCA
A710	TGTGACCA

i7 Index PCR Primer	Index Sequence
A711	AGGGTCAA
A712	AGGAGTGG

i5 Index PCR Primer	Index Sequence
A501	TGAACCTT
A502	TGCTAAGT
A503	TGTTCTCT
A504	TAAGACAC
A505	CTAATCGA
A506	CTAGAACA
A507	TAAGTTCC
A508	TAGACCTA

Illumina Amplicon Viewer

Upon completion of MiSeq sequencing, your data is automatically analyzed with MiSeq Reporter and can be visualized using the Illumina Amplicon Viewer. Amplicon Viewer has been designed and developed for off-instrument visualization and analysis of TruSeq Custom Amplicon Library Preparation (TSCA) data. Amplicon Viewer allows you to view data (including coverage, Q-score, variant call score, etc.) from multiple MiSeq amplicon runs simultaneously and interactively. You can also export custom reports based on selected samples/targets/variants.

Amplicon Viewer requires MiSeq Reporter data as input. You can download the Amplicon Viewer through your MyIllumina account. For more information about this software, see www.illumina.com/help/amplicon_viewer/default.htm.

To view a video demonstration of how to use the Illumina Amplicon Viewer, go to TruSeq Custom Amplicon Library Preparation Kit support page and click the Training tab.

Technical Assistance

For technical assistance, contact Illumina Technical Support.

Table 5 Illumina General Contact Information

Illumina Website	www.illumina.com
Email	techsupport@illumina.com

Table 6 Illumina Customer Support Telephone Numbers

Region	Contact Number	Region	Contact Number
North America	1.800.809.4566	Italy	800.874909
Austria	0800.296575	Netherlands	0800.0223859
Belgium	0800.81102	Norway	800.16836
Denmark	80882346	Spain	900.812168
Finland	0800.918363	Sweden	020790181
France	0800.911850	Switzerland	0800.563118
Germany	0800.180.8994	United Kingdom	0800.917.0041
Ireland	1.800.812949	Other countries	+44.1799.534000

MSDSs

Material safety data sheets (MSDSs) are available on the Illumina website at www.illumina.com/msds.

Product Documentation

Product documentation in PDF is available for download from the Illumina website. Go to www.illumina.com/support, select a product, then click **Documentation & Literature**.

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